

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

# The Presence and Significance of the Pi Class Glutathione S-Transferase Isoenzyme in Cerebrospinal Fluid during the Course of Meningitis in Children

SHERON WYLIE-MODRO, DAPHNE E HOLT, DAVID HARVEY, AND ROSALINDE HURLEY

*The Karim Centre for Meningitis Research, RPMS Department of Paediatrics and Neonatal Medicine, Queen Charlotte's and Chelsea Hospital, Goldhawk Road, London W6 0XG, United Kingdom*

## ABSTRACT

A rise in the concentration of the Pi class isoenzyme of glutathione S-transferase (GST) in the cerebrospinal fluid (CSF) during meningitis may be an early indicator of inflammation and cell damage. Pi class GST concentrations were measured in 48 samples of CSF from 46 children with proven or suspected meningitis using a commercially available immunoassay. Forty-four fetal brain samples were assayed by isoelectric focusing to determine the nature and number of isoenzymes likely to be released. Twenty-four percent of children had measurable amounts of the isoenzyme in their CSF during the initial stages of the disease. One child, for whom CSF samples were taken pre-, mid-, and post-antibiotic treatment, had measurable Pi class GST in the CSF only in the mid-treatment sample, when bacterial lysis and inflammation are likely to be at their peak. Where follow-up data were available, two of three children with measurable Pi class GST in their CSF at the

initial stages had recordable disabilities at 5 y of age compared with 4 of 11 of those in whom no Pi class GST was detected. Two proteins analogous to Pi class GST were detected in frozen brain tissue, but only one was active with a known substrate; only the active protein was seen in fresh tissue. We conclude that 1) initial high levels of CSF Pi class GST may be an indicator of the severity of inflammation and thus of prognostic significance and 2) only one Pi class GST occurs in brain tissue. (*Pediatr Res* 42: 232-236, 1997)

## Abbreviations

CSF, cerebrospinal fluid  
GST, glutathione S-transferase  
IEF, isoelectric focusing  
Mbrb, monobromobimane

The GST are a group of detoxicating isoenzymes that catalyze the conjugation of glutathione to a range of electrophilic compounds. The cytosolic isoenzymes are characterized by their electrophoretic mobility and their amino acid composition into Alpha, Mu, Pi, and Theta classes (1). Alpha and Pi class GST isoenzymes occur in high concentrations in various tissues, and it has been suggested that a rise in their concentration in body fluids may be an early marker of cell damage (2). For example, biliary epithelial cells contain high concentrations of Pi class GST and, if damaged, may release or actively secrete the isoenzyme into the bile from the epithelial cells lining the bile ducts (3). Elevated biliary levels of Pi class GST are associated with biliary obstruction, cholangiocarcinoma, and liver transplant rejection (Biotrin International, personal communication).

The Pi class isoenzyme of GST is localized in the brain to the choroid plexus (4, 5) and to the ventricular lining cells (4).

Its localization at the sites of the blood-CSF barrier and blood-brain barrier suggests that it may be released or secreted into the CSF under conditions of membrane inflammation and cell damage, such as occur in the course of meningitis. We describe here the assay of Pi class GST in the CSF of infants and children with meningitis to determine whether the isoenzyme is released from inflamed cells and to assess any possible relationship between such an event and the outcome of the disease.

It has been suggested that more than one Pi class isoenzyme exists in some tissues (5). We analyzed samples of fetal brain to determine the possible occurrence of multiple isoenzymes of Pi class GST and to investigate the possibility that one or more of these could leak or be secreted into the CSF after brain damage. The Pi class isoenzyme is detectable in human brain from the 12th wk of gestation (6).

## METHODS

**Sample collection.** The samples of CSF examined were collected in the course of a national survey of infantile meningitis (7). Consultant microbiologists were asked to provide samples of CSF, surplus to requirements after laboratory

Received May 22, 1996; accepted April 7, 1997.

Correspondence and reprint requests: Dr. Daphne E Holt, The Karim Centre for Meningitis Research, Queen Charlotte's and Chelsea Hospital, Goldhawk Road, London W6 0XG, UK.

Supported in part by REMEDI (Bath, Avon).

ination, from children under 1 y of age who were diagnosed with meningitis. The samples were stored at  $-20^{\circ}\text{C}$ . Forty-eight samples of CSF from 46 children with proven or suspected meningitis (Table 1) were analyzed. Thirty-nine children had bacteria cultured from their CSF, whereas six had sterile CSF. In a further child from whom three CSF samples were taken at different times, the first sample contained culturable bacteria, and the remaining two were sterile. Those with sterile CSF had clinical signs suggestive of meningitis; however, in all but one case CSF Gram stains and white cell counts were not available. Developmental data at 5 y of age

were available for 21 of the 46 children. A further group of 11 samples of CSF were analyzed from children who were found subsequently not to be suffering from meningitis (Table 2).

Brain tissue was collected in accordance with the recommendations of the Polkinghorne Report. Specimens of brain from 41 fetuses of gestational age 17–34 wk were collected at autopsy at Queen Charlotte's and Chelsea Hospital. All tissue was stored at  $-20^{\circ}\text{C}$  until analysis; storage of bodies before autopsy was at  $4^{\circ}\text{C}$ . Three further specimens of brain obtained at autopsy from fetuses of 15-wk gestational age were analyzed before and after freezing to determine the effect of storage.

Table 1. CSF sample details from 46 children with proven or suspected meningitis

Sample no.	Organism	WBC	%PMN	RBC	Protein (g/l)	Glucose (nmol/l)	Comments
17	H Infl	<100	99	nk	nk	nk	
59	H Infl	>100	95	<5	nk	nk	
63	H Infl	>100	nk	nk	nk	2.6	
176	H Infl	>100	99	>100	nk	nk	
201	H Infl	>100	33	<5	nk	8.0	
214	H Infl	>100	nk	<50	nk	nk	
220	H Infl	nk	nk	nk	nk	nk	
243	H Infl	>100	68	>100	4.8	1.0	Yellow color
270	H Infl	nk	nk	nk	nk	nk	
325	H Infl	nk	nk	nk	nk	nk	—*
333	H Infl	>100	90	<50	7.0	2.1	
497	H Infl	nk	nk	nk	nk	nk	
518	H Infl	nk	nk	nk	nk	nk	
572	H Infl	>100	95	<5	7.0	1.7	
591	H Infl	nk	nk	nk	nk	nk	
246	GBS	>100	80	<50	4.5	0	Yellow color
13	Strep Pneu	>100	90	<50	1.6	5.6	
5	Strep Pneu	>100	75	<10	nk	2.0	
73	Strep Pneu	>100	98	nk	2.2	8.0	
89	Strep Pneu	<100	nk	<10	nk	nk	
119	Strep Pneu	<5	99	<1	1.0	4.8	
165	Strep Pneu	nk	nk	nk	nk	nk	
250	Strep Pneu	>100	95	<50	nk	nk	
256	Strep Pneu	>100	95	<100	nk	3.9	
573	Strep Pneu	nk	nk	nk	nk	nk	
330	Strep Pneu	>100	90	nk	8.0	nk	
332	Strep Pneu	nk	nk	nk	nk	nk	
207	Strep Pneu	nk	nk	nk	nk	nk	Very cloudy
558	Strep Pneu	nk	nk	nk	nk	nk	
84	N Men	>100	90	<1	4.5	0	
95	N Men	<10	90	<1	1.0	3.8	
103	N Men	nk	nk	nk	nk	nk	
145	N Men	<1	nk	<10	nk	4.1	
159	N Men	>100	95	<50	1.4	3.7	
118	N Men	>100	90	>100	1.6	1.3	
267	N Men	nk	nk	nk	nk	nk	
580	N Men	nk	nk	nk	nk	nk	
593	N Men	nk	nk	nk	nk	nk	Blood-stained
597	N Men	nk	nk	nk	nk	nk	
10	NBG	nk	nk	nk	nk	nk	
32	NBG	nk	nk	nk	nk	nk	
20	NBG	>100	60	>100	2.7	3.0	Blood-stained
222	NBG	nk	nk	nk	nk	nk	Latex negative
24	NBG	nk	nk	nk	nk	nk	Latex negative
26	NBG	nk	nk	nk	nk	nk—*	Latex positive H Inf
27	NBG	nk	nk	nk	nk	nk	Latex negative
37	NBG	nk	nk	nk	nk	nk	Latex negative
47	NBG	nk	nk	nk	nk	nk	Latex negative

not known; NBG = no bacterial growth; GBS = group B streptococci; N Men = *Neisseria meningitidis*; H Infl = *Haemophilus influenzae*; Strep Pneu = *Streptococcus pneumoniae*; WBC = white blood cells; RBC = red blood cells; PMN = polymorphonuclear leukocytes.

Table 2. CSF sample details from 11 children who did not suffer from meningitis

Sample no.	Age of child (y)	WBC	RBC	Protein (g/L)	Glucose (nmol/L)
502	11/12	0	0	1.0	nk
517	7/12	0	0	0.1	nk
530	1/12	0	0	0.18	nk
531	8/12	0	0	0.4	2.6
78	3/12	0	0	0.4	nk
213	1 d	0	0	0.48	2.0
280	8/12	0	0	0.23	3.4
346	5/12	3	0	0.22	3.9
477	7/12	0	0	0.16	3.5
483	2/12	0	0	0.24	3.5
535	7/12	1	0	0.15	3.8

nk = not known; WBC = white blood cells; RBC = red blood cells.

**Sample processing.** Samples of CSF were analyzed without further processing. Brain specimens were thawed, weighed, and homogenized in three volumes of sodium phosphate buffer (4°C, 10 mM, pH 7) using six strokes of a hand-held homogenizer. The homogenates were centrifuged at  $4500 \times g$  (4°C, 10 min) to remove cell debris, and the resulting supernatants were further centrifuged at  $90\,000 \times g$  (4°C, 1 h) for recovery of the cytosolic fractions. Cytosols not analyzed immediately were held at -20°C. Protein concentration was determined in all cytosolic fractions using the method of Lowry (8).

**Assay of Pi class GST.** The concentration of Pi class GST was determined in CSF samples (20 µL) using immunoassay kits (HepKit-Pi) kindly supplied by Biotrin International (Co Dublin, Ireland), and following the protocol supplied.

**IEF.** All IEF was carried out using a multiphor II horizontal electrophoresis system cooled to 10°C (LKB, Pharmacia Biotech, St Albans, Herts, UK). Gels (1.0 mm) were cast using 29.1% acrylamide containing 0.9% bisacrylamide and with the addition of 1.5 mL of ampholines (pH range 3.5–10) on GelBond PAG film (Pharmacia). Diluted samples of brain cytosol (10 µL) were applied on applicator strips together with IEF standards (pI range 4.7–10.6; BDH-Merck, Poole, Dorset, UK) and focused for 75 min at 50 mA current to a maximum of 1500 V and 30 Watts power. Anode and cathode solutions were phosphoric acid (1 M) and sodium hydroxide (1 M), respectively. The presence of the Pi class GST isoenzyme was determined by specific enzyme activity or by immunoblotting.

**Mbrb staining.** Active GST isoenzymes were visualized by staining with Mbrb, a specific substrate that reacts with thiols to give fluorescent products (9). Immediately after IEF, the gel was removed from the backing film and washed for 2 min in potassium phosphate buffer (1 M, pH 6.5, room temperature) containing glutathione (0.5 mM). After washing, the gel was incubated for 30 s in the same buffer with the addition of Mbrb (0.5 mM; Sigma Chemical Co.-Aldrich Company, Poole, Dorset, UK). The gel was rinsed rapidly to remove unbound products. Bound fluorescence was recorded on Polaroid film (type 677) after excitation at 400 nm.

**Immunoblotting.** Immediately after IEF the gel was removed from the backing film and blotted (160 mA, 45 min, room temperature; Novoblot, LKB-Pharmacia) onto nitrocellulose membrane (Hybond-ECL; Amersham International, Lit-

tle Chalfont, Bucks, UK) in a transfer buffer containing glycine (39 mM), Trizma base (48 mM), SDS (0.037%), and methanol (20%). Nonspecific binding sites were blocked by overnight incubation at 4°C in PBS containing Tween 20 (0.1%, pH 7.2, PBS/Tween) and powdered milk (5%). After removal of the blocking solution, the primary antibody was added (rabbit anti-human Pi class GST; Biotrin International) as a 1:1000 dilution in PBS/Tween containing 3% powdered milk, and incubation was continued for a further hour at room temperature with continuous agitation. The membrane was washed with PBS/Tween and incubated with a 1/1000 dilution of the secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase; Bio-Rad Laboratories, Hemel Hempstead, Herts) for 1 h at room temperature, again with continuous agitation. After two further washings, Pi class GST was visualized using an enhanced chemiluminescence Western blotting detection kit (Amersham International). The luminescence emission was detected by exposure to blue light-sensitive autoradiography film (ECL-Hyperfilm, Amersham International).

**Data analysis.** Concentrations of Pi class GST are expressed as nanograms/mL of fluid with means and 95% confidence intervals as appropriate. Fisher's exact test was used to compare proportions.

## RESULTS

The Hepkit-Pi had a limit of detection of 30 ng/mL. Of 45 children with proven or suspected meningitis, 11 (24%) had measurable amounts of Pi class GST in their CSF (Table 2). Ten of the 11 children had proven meningitis, and in one the CSF was sterile. The presence of the isoenzyme in the CSF did not relate to the type of infecting bacteria. A single CSF sample was taken from each of the 45 children before treatment commenced, but three samples were collected from another child; one sample before commencement of antimicrobial therapy, one during therapy, and one at the end of the course. The first sample was culture-positive for *Haemophilus influenzae*, the second grew no bacteria but was positive for *H. influenzae* by latex testing (Welcogen, Wellcome Diagnostics), and the third was negative by both criteria (our unpublished observations). The second of these samples contained 65 ng/mL of Pi class GST (sample 326, Table 3), the others were negative. Four of the samples assayed were visibly blood-stained, were xanthochromic, and these had concentrations of isoenzyme above the upper limit of the assay (265 ng/mL). These samples were excluded from any further analysis on the basis that erythrocytes contain high levels of Pi class GST (2). The mean concentration in positive samples uncontaminated with blood ( $n = 8$ ) was 107.8 ng/mL, confidence interval 58.1–157.5. No Pi class GST was detectable in the CSF of the 11 children who were found not to be suffering from meningitis.

Of 21 children for whom follow-up information was available, 4 of 15 (27%) with no detectable Pi class GST in their CSF had disabilities at 5 y of age (squint, hearing loss, mild monoplegia), and two of three (67%) with detectable levels of CSF Pi class GST had disabilities (profound hearing loss, bilateral squint). Another disabled child with a high level

Table 3. Concentration of Pi class GST in the CSF of children with proven or suspected meningitis

Sample no.	Age of child (y)	Five-year follow-up	Infecting organism	Concentration of Pi class GST (ng/L)	Appearance
17	11/12	OK	H Infl	90	Colorless/clear
214	1/12	Profound hearing loss	H Infl	120	Colorless/clear
243	2/12	Mild hearing loss	H Infl	>265	Straw-colored/clear
246	1 wk	Lost to F/U	GBS	>265	Straw-colored/clear
89	1 d	Spina bifida	Strep Pneu	260	Colorless/clear
330	?	Lost to F/U	Strep Pneu	87.5	Colorless/clear
507	12/12	Lost to F/U	Strep Pneu	60	Colorless/cloudy
318	4/12	Bilateral squint	N Men	150	Colorless/clear
593	12/12	Lost to F/U	N Men	>265	Blood-stained
597	12/12	Lost to F/U	N Men	30	Colorless/clear
320	2/12	Cerebral palsy	NBG	>265	Blood-stained
326	10/12	Lost to F/U	NBG	65	Colorless/clear

F/U = follow-up; NBG = no bacterial growth; GBS = group B streptococci; N Men = *Neisseria meningitidis*; H Infl = *Haemophilus influenzae*; Strep Pneu = *Streptococcus pneumoniae*.

CSF-Pi class GST had contracted meningitis as a complication of spina bifida, which may itself have led to raised GST levels. There was no significant difference between these proportions. On IEF and subsequent immunoblotting with anti-Pi class GST antibody, brains from fetuses of all ages showed two major protein bands with isoelectric points of pI 4.8 and 4.95 (for two examples of 19 and 20 wk of gestation, respectively, see Fig. 1A, lanes 1 and 2 and 3 and 4). Pi class GST has an pI of 4.8 (10). In some specimens, additional faint bands were seen at pI values up to 5.65. Staining with Mbrb indicated that only one of the bands (pI 4.8) was an isoenzyme active with this substrate (data not shown). Analysis of three fresh brain specimens from fetuses of 15-wk gestational age, obtained at autopsy immediately after termination of pregnancy, showed only one protein band on immunoblotting of pI 4.8. Figure 1C, lanes 9-13, shows Pi class GST from one of these brains, and this was confirmed as an active enzyme by staining with Mbrb. Further analysis of these brains indicated that changes in the isoenzyme such that Mbrb no longer bound to the active site were occurring on storage of the whole tissue at -20°C, but when held as a cytosol at the same temperature. Figure 1B, lanes 5 and 6 and 7 and 8, shows the cytosolic fraction prepared from two of these brains.

## DISCUSSION

Release of the Pi class isoenzyme of GST from injured cells, either by simple leakage or active secretion, is postulated for other organs (3), and its location and heavy concentration in the choroidal epithelium (6) gives weight to the hypothesis that the same may be true in the brain. Pi class GST was detected in the CSF of 24% of children with meningitis (Table 3). This suggests that Pi class GST is not invariably released or secreted from cells into the CSF in the early stages of the disease. In experimental *H. influenzae* meningitis antibiotic-induced bacterial lysis enhances the inflammatory response (11). In one child with *H. influenzae* meningitis and from whom CSF samples were obtained before, during, and after antibiotic treatment, detectable Pi class GST levels were only in the sample taken during treatment, in which bacterial lysis along with inflammation and thus likely cell damage would be at its peak. As the untreated disease progresses, it is possible that cells may suffer damage such that a threshold is passed at which Pi class GST is released or secreted. Children with detectable Pi class isoenzyme in pretreatment CSF samples may have a more advanced inflammatory response and thus a poorer prognosis. Sixty-seven percent of children with mea-

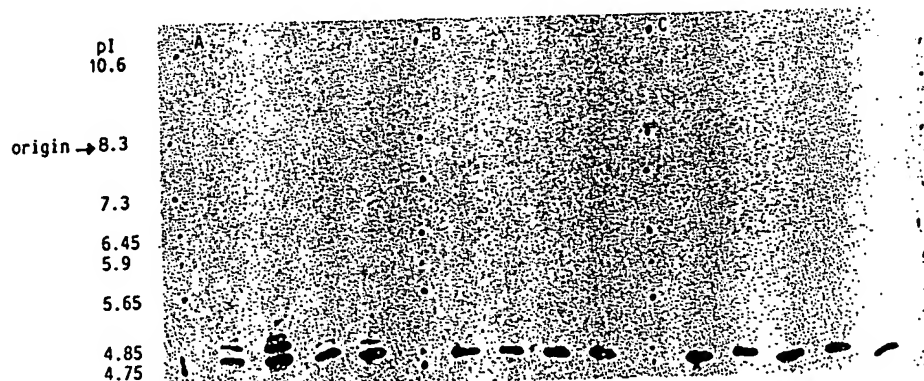


Fig. 1. IEF gel of (A) duplicate brain specimens from fetuses of 19- and 20-wk gestational age in which the tissue was stored at -20°C (lanes 1 and 2 and 3 and 4, respectively); (B) duplicate brains from two fetuses of 15-wk gestational age, frozen after preparation of the cytosolic fractions (lanes 5 and 6 and 7 and 8, respectively); and (C) five specimens of brain from a fetus of 15-wk gestational age analyzed without freezing (lanes 9-13). The Pi class GST was visualized by immunoblotting with anti-Pi class GST antibody, and the emitted light was captured on blue light-sensitive autoradiographic film. pI markers are shown on the left, and the origin is indicated (→).

surable levels of Pi class GST had recordable disabilities at 5 y of age compared with 27% of those without. However, the numbers are small and preclude any meaningful statistical analysis.

Two isoenzymes have been described in skeletal muscle with pI 4.8 and 4.5 (5). These forms had identical N-terminal amino acid sequences that were similar to Pi class GST. Fetal brain tissue stored at  $-20^{\circ}\text{C}$  before probing with anti-Pi class GST antibody also showed the appearance of multiple forms of the isoenzyme. Two major forms with slight differences in pI were detected; however, only one of these (pI 4.8) reacted with Mbrb. There were no differences in the pattern of bands that could be said to correspond to the gestational age of the fetuses. It is possible that each of the two forms of Pi class GST detected has a different substrate specificity. It is also possible that the isoenzyme may have been sufficiently structurally degraded during storage to destroy the active site but remain recognizable by the antibody. Fresh brain tissue contained only one isoenzyme (pI 4.8) on both immunoblotting and staining with a specific substrate. This observation indicates that, in second and third trimester fetal brain, only one form of Pi class GST exists and that the appearance of apparent multiple forms on immunoblotting is an artifact of tissue storage.

Location of Pi class GST in the choroid plexus and ventricular linings makes it a candidate for possible leakage or secretion into the CSF under conditions of cellular damage such as that occurring in meningitis. The data reported here show that Pi class GST can be detected in some cases of meningitis, and when it is detectable, the children appear to

have a poorer prognosis although, in this instance, statistical significance was not attained. A countrywide survey of children with meningitis is in progress in which samples of CSF will be analyzed to further test this hypothesis.

**Acknowledgments.** The authors gratefully acknowledge the help of Dr. Gillian Gau and Dr. Phillip Bennett in collecting the brain tissue. We also thank Biotrin International for their generous gift of Hepkit-Pi assay kits.

## REFERENCES

1. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR, Pickett CB, Sato K, Widerstein M, Wolf CR 1992 Nomenclature for human glutathione transferases. *Biochem J* 282:305-306
2. Beckett GJ, Hayes JD 1993 Glutathione S-transferases: biomedical applications. *Arch Clin Chem* 30:281-380
3. Howie AF, Hayes PC, Bouchier IAD, Hayes JD, Beckett GJ 1989 Glutathione S-transferase in human bile. *Clin Chim Acta* 184:269-278
4. Tsuchida T, Hruban RH, Carson BS, Phillips PC 1992 Colloid cysts of the third ventricle: immunohistochemical evidence for non neuroepithelial differentiation. *Hum Pathol* 23:811-816
5. Singh SV, Ahmad H, Kurosky A, Awasthi YC 1988 Purification and characterization of unique glutathione S-transferases from human muscle. *Arch Biochem Biophys* 264:13-22
6. Carder PJ, Hume R, Fryer AA, Strange RC, Lauder J, Bell JE 1990 Glutathione S-transferase in human brain. *Neuropathol Appl Neurobiol* 16:293-303
7. de Louvois J, Blackbourn J, Hurley R, Harvey D 1991 Infantile meningitis in England and Wales: a two year study. *Arch Dis Child* 66:603-607
8. Lowry OH, Rosenbrough NH, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275
9. Hulbert PB, Yakubu SI 1983 Monobromobimane: a substrate for the fluorimetric assay of glutathione transferase. *J Pharm Pharmacol* 35:384-386
10. Hayes JD, Pickett CB, Mantle TJ (eds) 1990 Nomenclature for GST. In: *Glutathione S-Transferases and Drug Resistance*. Taylor & Francis, London, pp xi-xiv.
11. Mertsola J, Ramilo O, Mustafa MM, Saez-Llorens X, Hansen EJ, McCracken GH 1989 Release of endotoxin after antibiotic treatment of Gram-negative bacterial meningitis. *Pediatr Infect Dis J* 8:904-906